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Original Article

Further investigation on nuclear transplantation in different orders of teleost: the combination of the nucleus of Tilapia *(Oreochromis nilotica)* and the cytoplasm of Loach *(Paramisgurnus dabryanus)*

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ABSTRACT The nucleus of a blastula cell from Tilapia (*Oreochromis nilotica,* family Cichlidae, order Perciformes) was transplanted into an enucleated egg of Loach (*Paramisgurnus dabryanus,* family Cobitidae, order Cypriniformes). From among 3747 nucleo-cytoplasmic hybrid (NCH) eggs two NCH larval fish (0.05%) were obtained; one died on the 6th day and the other died on the 12th day after the operation. Morphological examinations showed that both NCH larval fish had developed normally with an opened mouth except they could not take food after complete utilization of their egg yolk on the 5th day of development. The possible mechanisms for obtaining such inter-order NCH larval fish are discussed. This is the first report indicating that inter-order NCH larval fish can be obtained in spite of their evolutionary divergence.

KEY WORDS: *nuclear transplantation, different orders, teleost*

Introduction

Our previous papers reported that nucleo-cytoplasmic hybrid (NCH) adult fishes can be obtained from the combination of the nucleus and the cytoplasm of the fishes of different varieties belonging to the same species (Crucian carp, *Carassius auratus* wild type and Goldfish, *Carassius auratus*, domestic type, Tung and Yan, 1985); of different genera (Common carp, *Cyprinus carpio*, genus Cyprinus Linnaeus and Crucian carp, *Carassius auratus*, wild type, genus Carassius Jarocki, Tung, 1980; Yan *et al.*, 1984a; Yan *et al.*, 1986) and of different subfamilies (Grass carp, *Ctenopharyngodon idellus*, subfamily Leucinae and Blunt-snout bream, *Megalobrama amblucephala*, subfamily Abramidinae, Yan *et al.*, 1984b, 1985). In the case of different families, NCH larval fishes were obtained from the combination of the nucleus of the Loach (*Paramisgurnus dabryanus*, family Cobitidae) and the cytoplasm of Goldfish (*Carassius auratus*, domestic type, family Cyprinidae) and vice versa (Yan *et al.*, 1990). However, neither NCH adult nor larval fishes were obtained when the nucleus of the fish of one order (Tilapia, *Oreochromis nilotica*, family Cichlidae, order Perciformes) was transplanted into the enucleated egg cytoplasm of the fish of another order (Goldfish, *Carassius auratus*, domestic type, family Cyprinidae, order Cypriniformes) (Yan *et al.*, 1990). Instead, in this combination, only NCH embryos of early stages (blastula and early gastrula) developed.

Our tentative explanation for the failure to obtain NCH larval or adult fishes in this inter-order combination was that there exist serious developmental incompatibilities between the nucleus and the cytoplasm of those two species (Yan *et al.*, 1990). So far, it has been very difficult to identify what kinds of incompatibility are involved in that combination. However, the chromosome number of the two species of distantly related fishes are quite different, *i.e.*, 44 (2N) in the nucleus type fish (Tilapia, *Oreochromis nilotica*), and 100 (2N) in the cytoplasm type fish (Goldfish, *Carassius auratus*, domestic type). This difference seemed to be one of the possible essential factors which might cause serious incompatibilities between the Tilapia nucleus and the Goldfish cytoplasm in the NCH egg and hence influence the developmental capacity of the NCH eggs (Yan *et al.*, 1990).

Abbreviations used in this paper: NCH, nucleo-cytoplasmic hybrid

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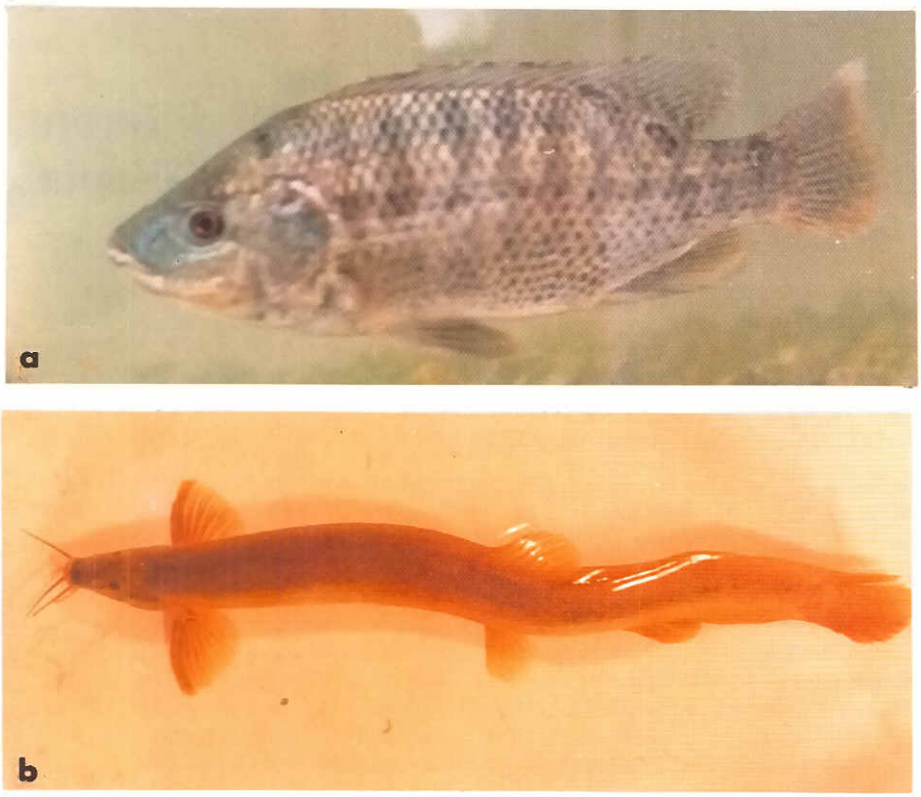


Fig. 1. Nuclear transplantation between the fishes of different orders. **(a)** *Tilapia* (Oreochromis nilotica, *family Cichlidae, order Perciformes, 2N=44).* **(b)** *Loach* (Paramisgurnus dabryanus, *family Cobitidae, order Cypriniformes, 2N=48).*

In order to examine this possibility, it was necessary to conduct nuclear transplantation experiments between two species belonging to different orders but possessing a similar chromosome number. The transplantation of nucleus of Tilapia (*Oreochromis nilotica*, family Cichlidae, order Perciformes, 2N=44) (Fig. 1a) into the enucleated egg cytoplasm of Loach (*Paramisgurnus dabryanus*, family Cobitidae, order Cypriniformes, 2N=48) (Fig. 1b) seemed to be an appropriate combination for the present purpose. There is a difference of only four chromosomes in those two distantly related fishes, whereas there was a difference of fifty-six chromosomes in the combination between the fishes belonging to the same two orders but two different families in our previous experiment (Yan *et al.*, 1990). In addition, this particular combination permits the identification of the Tilapia genome because of the presence of two long chromosomes of Tilapia which are excellent markers for identifying the origin of the NCH embryos (Fig. 3e).

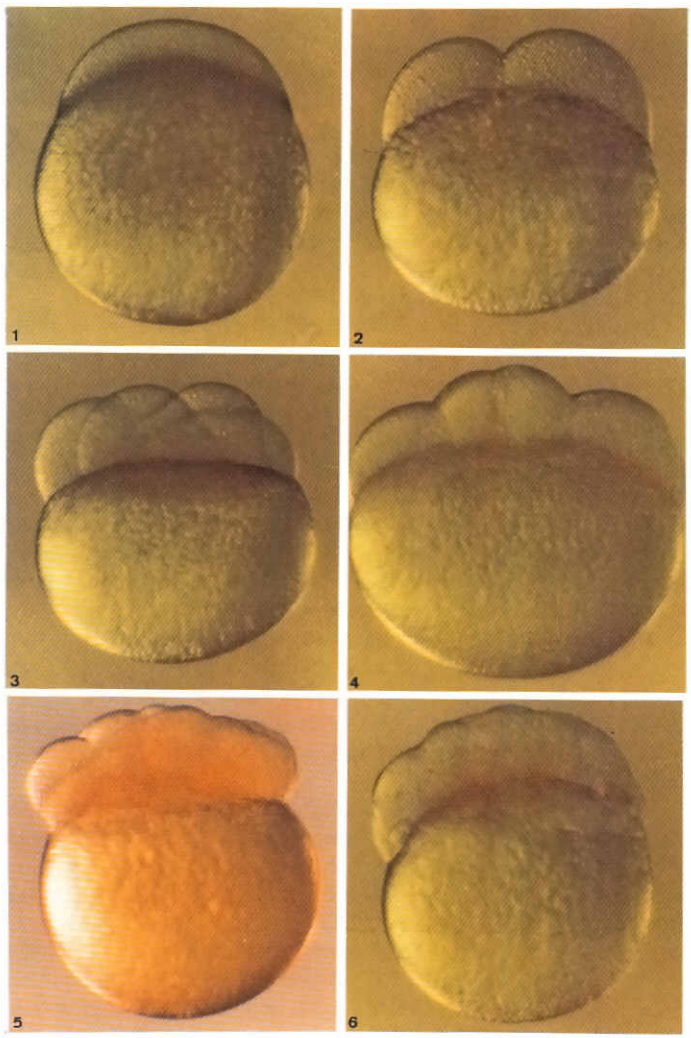
Results

A total of 3747 enucleated Loach eggs were transplanted with the nuclei of Tilapia blastula cells (Table 1). Of these NCH eggs, 2831 (75.5%) developed into blastulae, 78 (2.07%) into early gastrulae, 21 (0.58%) into late gastrulae, 14 (0.37%) into neurulae, 5 (0.13%) into tail bud embryos, 5 (0.13%) into heart beating embryos and 2 (0.05%) into larval fishes; one NCH larval fish died on the 6th day and the other died on the 12th day after nuclear transplantation. Fig. 2, 1-16 show the development of one NCH fish in this experiment.

The developmental rate of the NCH eggs is similar to that of Loach eggs but much faster than that of Tilapia eggs. For example, the egg yolk was completely utilized by the NCH larval fish by the fifth day (125.5 h) of its development (Fig. 2, 16), while the Tilapia larval fishes still possessed abundant egg yolk on the sixth day of development (Fig. 3f). However, the NCH larval fish did not begin to take food on the 6th day even though the mouth and digestive organs seemed to be well developed. The normal Loach larval fishes of the same age begin to catch food, whereas Tilapia larval fishes continue to utilize egg yolk as their nutritional source until the 21st day when their egg yolk completely disappears, their mouths open and they initiate feeding.

The morphological examination of both NCH larval fishes and Loach larval fishes showed that no embryonic external gills or oral barb rudiments were detected in the NCH larval fish (Fig. 3a) whereas five pairs of embryonic external gills and three pairs of oral barb rudiments were clearly detectable in the Loach larval fish at similar developmental stages (Fig. 3b, only two pairs out of the total five pairs of embryonic external gills of the larval fish are visible in the photograph).

Fig. 2. Early development of a nucleo-cytoplasmic hybrid (NCH) embryos obtained from the combination of the nucleus of Tilapia *(Oreochromis nilotica,* family Cichlidae, order Perciformes, 2N=44) and the cytoplasm of Loach (*Paramisgurnus dabryanus,* family Cobitidae, order Cypriniformes, 2N=48). **(1)** *1-cell,* **(2)** *2-cell,* **(3)** *4-cell,* **(4)** *8-cell,* **(5)** *16-cell,* **(6)** *32-cell,* **(7)** *64-cell,* **(8)** *early blastula,* **(9)** *late blastula,* **(10)** *middle gastrula,* **(11)** *late gastrula,* **(12)** *eye cup stage,* **(13)** *muscle contraction stage (23 h),* **(14)** *47 h,* **(15)** *70 h,* **(16)** *125.5 h.*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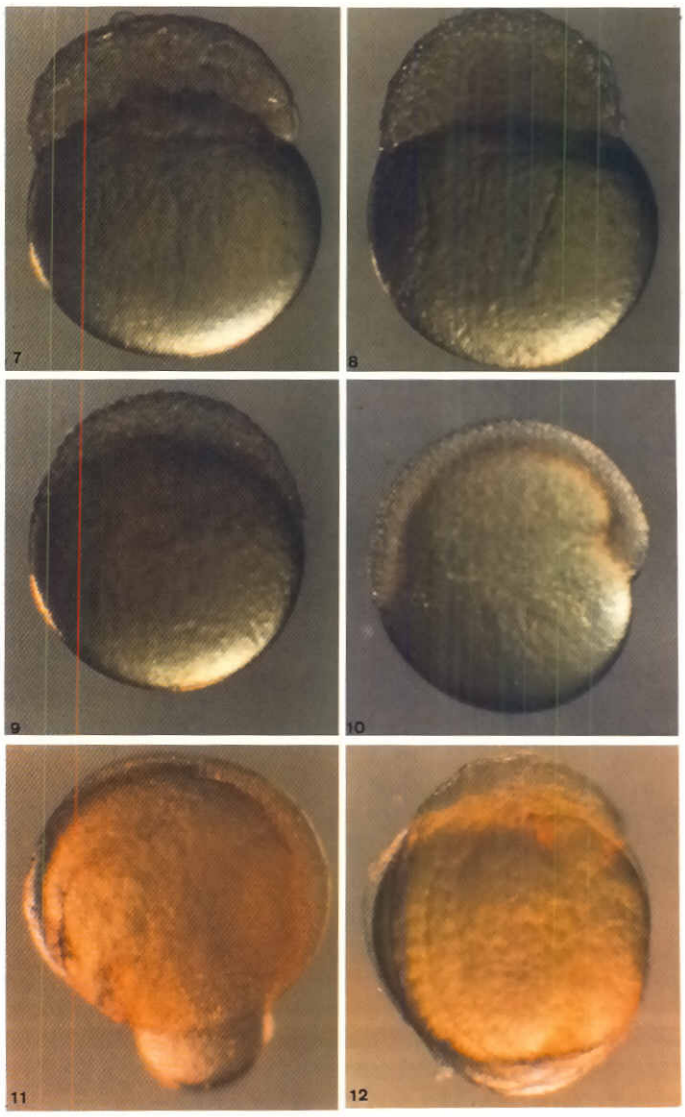
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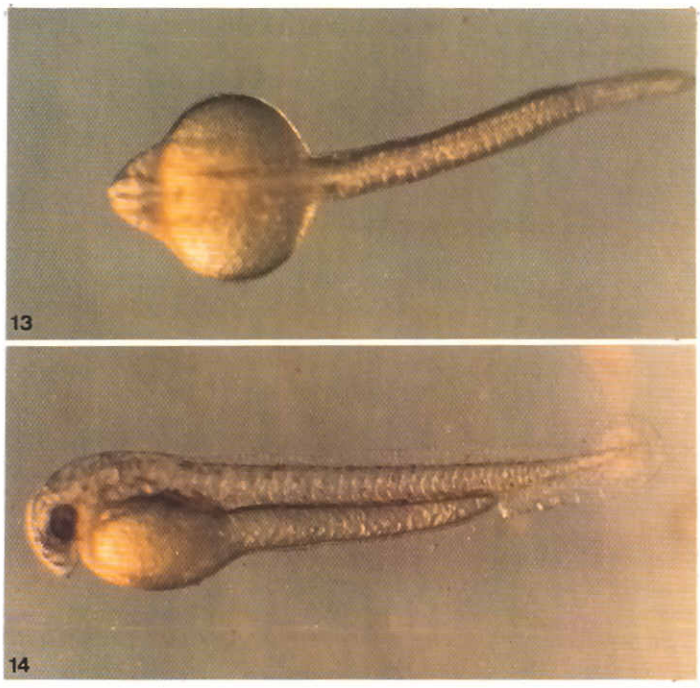
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The two NCH larval fishes seemed to develop normally until the 5th day of culture. They developed brain, nervous, circulatory and digestive systems as well as a mouth, body pigmentation and body fins, but their eyes seemed to be smaller than those of Loach and Tilapia fishes (Fig. 2, 16). The NCH larval fishes initially swam lively in the fresh water of the culture. Later, however, due to the non-functional mouth and the absence of egg yolk for supporting their further growth, one NCH larval fish died on the sixth day and the other became very weak but remained alive until the 12th day.

The metaphase chromosome patterns of 14 NCH and 21 Loach early gastrulae were examined respectively (the remaining numbers of NCH gastrula had to be kept alive to observe their further development). The two long marker chromosomes of Tilapia were identified in all the metaphase chromosome patterns of NCH gastrulae examined and no obvious abnormal karyotypes were observed. Fig. 3e showed the metaphase chromosome pattern of one NCH gastrula. Therefore, it is certain that the genome of the NCH gastrula is that of the nuclear donor, Tilapia.

Discussion

It has been well established that the embryonic nucleus and the enucleated egg cytoplasm of fishes from different subfamilies, genera and varieties can be combined by the nuclear transplantation technique and that the resultant NCH eggs can in some cases complete their development from the early embryo stage to the adult stage. It has also been shown, however, that the more distantly related the species used in combination, the more serious the developmental incompatibilities revealed in post-blastula development (Yan *et al.*, 1990). For example, in the inter-order combination when the nucleus of Tilapia (order Perciformes) was combined with the egg cytoplasm of Goldfish (order Cypriniformes), the percentage of the NCH blastula obtained was 53.7% (Yan *et al.*, 1990). This percentage is not significantly different from that obtained in the inter-subfamily combination (nucleus of Grass carp, subfamily Leucinae + egg cytoplasm of Blunt-snout bream, subfamily Abramidinae, 43.8%) (Yan *et al.*, 1985) and the inter-genus combination (nucleus of Common carp, genus Cyprinus + egg cytoplasm of Crucian carp, wild type, genus Carassius Jarocki, 58.7%) (Yan *et al.*, 1985). However, in the inter-order combination, the NCH embryos developed no further than the blastula or early gastrula stages (Yan *et al.*, 1990), while in the inter-subfamily and inter-genus combinations, some adult NCH fishes were obtained (Yan *et al.*, 1985, 1986). We assume that the factors responsible for initiating the development of NCH eggs in the inter-genus, inter-subfamily, inter-family and even in inter-order combinations should not be different. If so, why do the NCH eggs obtained in inter-order combination develop only into early embryonic stages? Is the reason only because the fishes used in inter-order combination are so distantly related? We think not, because we found that in the inter-order combination the chromosome number of the nucleus-donor fish and that of the cytoplasm-recipient fish is considerably different; Tilapia has 44 chromosomes (2N) and Goldfish has 100 chromosomes (2N) (Yan *et al.*, 1990), whereas in the inter-subfamily combination, Grass carp has 48 chromosomes (2N) and Blunt-snout bream has 48 chromosomes (2N) (Yan *et al.*, 1985); in the inter-genus combination, common carp has 100 chromosomes (2N) and Crucian carp has 100 chromosomes (2N) (Tung, 1980). Thus, the chromosome number of both the nucleus-donor fish and the cytoplasm-recipient fish in both the inter-subfamily and inter-genus combinations were the same.

According to Tung *et al.* (1973), when the nucleus of Goldfish (*Carassius auratus*, subfamily Cyprinidae, order Cypriniformes, 2N=100) was transplanted into the enucleated egg cytoplasm of Chinese bittering (*Rhodeus sinensis*, subfamily Acheilognathinae, order Cypriniformes, 2N=46), neither NCH larval nor NCH adult fishes were obtained; only some NCH embryos at early developmental stages developed. If this inter-subfamily combination is compared with the other inter-subfamily combination between Grass carp and Blunt-snout bream (Yan *et al.*, 1985), it is obvious that the difference in the chromosome number of the former combination is much larger than that of latter combination.

Thus, we proposed previously (Yan, 1989; Yan *et al.*, 1990) that the difference in chromosome number between the nucleus-donor and the recipient enucleated egg cytoplasm provides one of the essential factors influencing the extent of development of NCH eggs irrespective of how far the two species of fishes are taxonomically classified.

In the studies presented in this paper, the nucleus and cytoplasm were also combined from the fishes of different orders but with chromosome numbers of minor difference; Tilapia has 44 chromosomes (2N) and Loach has 48 chromosomes (2N). The survival percentage of NCH blastula is 75.5% whereas that of the previous inter-order combination of Tilapia nucleus and Goldfish cytoplasm was 53.7% to 71.8%. The survival percentage of NCH gastrula in the present combination is 2.07% to 0.58%, while that of the combination of Tilapia nucleus and Goldfish cytoplasm was 0.15%. Finally, two NCH larval fishes were obtained in the present combination, whereas no NCH larval fishes were obtained in the combination of Tilapia nucleus and Goldfish cytoplasm.

This comparison demonstrates that the developmental potential of the NCH eggs obtained from the combination of Tilapia nucleus and Loach cytoplasm is much higher than that of the NCH eggs obtained from the combination of Tilapia nucleus and Goldfish cytoplasm. In both combinations, the nuclei were taken from the same species of fish (Tilapia) but the cytoplasm was obtained from fish of the same order (Cypriniformes) but different families (Loach or Goldfish), so the taxonomic relationship between the nucleus-donor and the cytoplasm-recipient fish species in either combination is similar but the chromosome numbers are significantly different between the two cytoplasmic-recipient fishes, Loach (2N=48) and Goldfish (2N=100). In view of the similar results

Fig. 3. Morphological comparison of the nucleo-cytoplasmic hybrid (NCH) larval fish obtained from the combination of the nucleus of Tilapia (*Oreochromis nilotica*, family Cichlidae, order Perciformes, 2N=44) and the cytoplasm of Loach (*Paramisgurnus dabryanus*, family Cobitidae, order Cypriniformes) and the larval fish of Loach. (a) *A 46.6-h NCH larval fish without embryonic external gills and oral barb rudiments.* **(b)** *A 47-h larval fish of Loach. The big arrow indicates its embryonic external gills and the small arrow its oral barb rudiments.* **(c)** *A 125.5-h NCH larval fish. No barb appeared.* **(d)** *A 120-h Loach larval fish. Two pairs of oral barbs are present (arrows) and the third pair of barbs is located underneath the front part of the mouth and is not shown in this picture.* **(e)** *A metaphase of a NCH gastrula. Arrows indicate the long marker chromosomes of Tilapia (2N=44).* **(f)** *A Tilapia larval fish at 150 h after fertilization.*

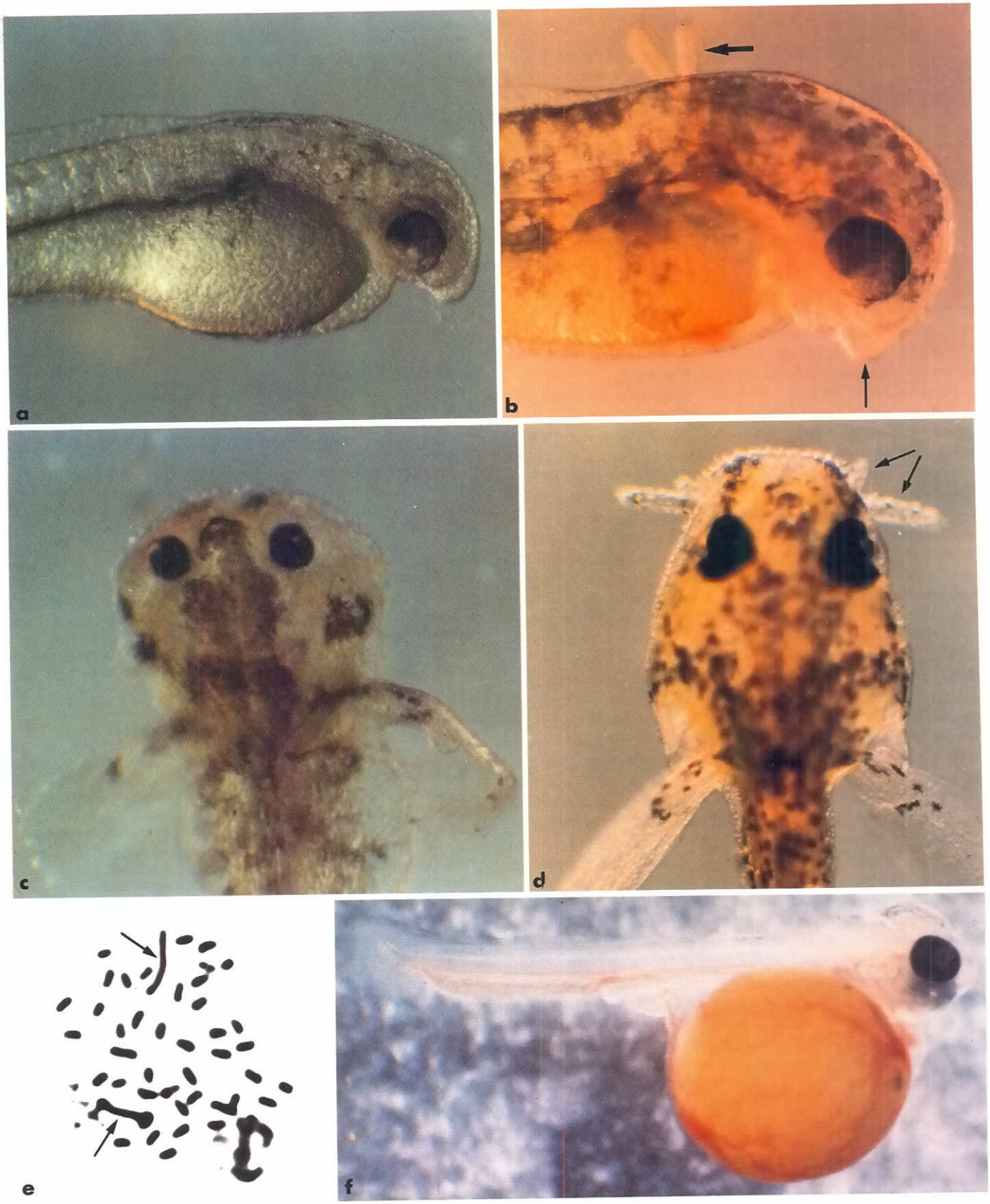
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TABLE 1

SURVIVAL PERCENTAGE AT EACH DEVELOPMENTAL STAGE OF NUCLEAR TRANSPLANTED EGGS OF TILAPIA NUCLEUS AND LOACH CYTOPLASM

Total No. of transplanted eggs	Number attaining stage of						
	blastula	early gastrula	late gastrula	neurula	tail bud	heart beat	larval fish
3747 (100%)	2831 (75.5%)	78 (2.07%)	21 (0.58%)	14 (0.37%)	5 (0.13%)	5 (0.13%)	2 (0.05%)

obtained in the inter-genus and inter-subfamily combinations, it could be argued that the incompatibility of the different genomic components of the transplanted nuclei and its counterpart – recipient egg cytoplasm is one of the essential factors influencing the developmental potential of different NCH eggs.

It was only in the present inter-order combination of Tilapia and Loach that the NCH larval fishes – though only in 2 cases – were obtained. In the combination of Tilapia with Goldfish none developed into larval fishes. Therefore, NCH eggs in the Tilapia and Loach combination have the potential to complete the whole morphogenetic process to larval stage in spite of the natural developmental incompatibility between the nucleus and cytoplasm of inter-order origin. However, there still exist some barriers in this combination which may prevent the NCH larval fishes from growing into adult fish, since the two NCH larval fishes were unable to survive without a further nutrition supply once their egg yolk was used up. We think that another important natural barrier that may prevent the further growth of the NCH larval fishes in this combination is the conspicuous difference in feeding behavior of larval fishes of the two species. The genotype of the NCH larval fish is that of the nucleus-donor type (Tilapia) and the egg yolk of Tilapia is not utilized until the 21st day of its development, at which time the larval fish starts to eat, while the developmental speed of the NCH eggs is according to the type of recipient egg (Loach). In Loach, the egg yolk of the larval fish is used up as early as the 5th to 6th days of development and thereafter they initiate feeding. In the NCH larval fishes (Tilapia-Loach), the yolk was utilized prior to the time that the larvae could initiate feeding and therefore they could not maintain further growth. Therefore, it is reasonable to conclude that the developmental feeding behavior of the Tilapia and Loach larval fish may prevent the NCH larval fish from growing into an adult.

The question still remains whether the natural evolutionary barriers between the distantly related fish species can be overcome in the NCH eggs, if the proper combination of fishes could be found. In order to answer this question, we are looking for candidate fishes of different families or orders which have not only similar chromosome number but also have a similar developmental feeding behavior. With such kinds of fish combinations it may be possible to obtain NCH larval fish that advance to older larval stages, and perhaps even into the adult stage.

Materials and Methods

Fish stocks and technique for nuclear transplantation

Fresh water cultured teleosts, the Tilapia and Loach were provided by the aquaculture facilities of the Institute of Developmental Biology, Academia Sinica, Beijing, the Guangxi Fisheries Institute, Nanning and the Fresh Water Fisheries Research Center, Wuxian, China. The methods for enucleating eggs and transplanting blastula cell nuclei were reported in a previous paper (Yan, 1989).

Chromosome examination

The technique for obtaining chromosome preparations in fish embryos was according to Yamazaki's method (Yamazaki, 1981).

Acknowledgments

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